ENZYME SYSTEMS INVOLVED IN NITROGEN METABOLISM IN GREEN PLANTS AND THEIR INFLUENCE ON YIELD AND GRAIN PROTEIN PRODUCTION IN WHEAT

by

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CHAPTER I

INTRODUCTION

At present wheat contributes a substantial proportion of the world food supply. However, rapidly increasing human populations have created the need for higher yielding cereal grains with higher protein quantities and qualities. The problem of nutrition in the world is related primarily to a shortage of protein. Better nutrition for the millions of people who rely upon cereals as their principal food may well depend upon the improvement of the inherent nutritional quality of the cereals.

The urgency of the world's protein crisis has engaged the concern of governments, international agencies and research foundations. Cereal grains are and will continue to be the world's dietary mainstay, not only for calories but for protein as well. It is clear, therefore, that a challenging and potentially fruitful area for priority action in dealing with the world's protein crisis is to increase the production of cereal grains, and to increase the quality and quantity of their protein by various biological, technological and chemical means. It is clear that the burden of closing the world's widening protein gap will fall largely on cereal producing capabilities.

The discovery of Opaque-2 mutant gene in corn by Mertz et al. in 1964 which increased the amount of lysine and tryptophan content in the endosperm has focused attention on the possibility that similar

genes could be found in all cereals. Since this discovery, there has been worldwide excitement and activity in research programs to identify genes for improved protein quality.

Protein formation in the wheat grain is a function of genetic factors interacting with soil and climate. Soil fertility is a major factor that may significantly influence the quantity of protein synthesized in the wheat grain. Nitrogen fertilization and nitrogen metabolism in the plant are directly involved in the increase of protein production.

It is well known that nitrate is the primary form of nitrogen available to most plants. The reduction sequence of nitrogen is initiated by the enzyme, nitrate reductase. This is the first enzyme in the nitrogen metabolic sequence and acts as a major control point for the supply of reduced nitrogen to the plant for protein synthesis (Beevers and Hageman, 1969).

Evidence of the existence of large inherent differences in the grain protein content in wheat was obtained by Middleton et al. in 1954. Since then an extensive study on the agronomic relations and inheritance of high protein in wheat have been reported. Haunold, Johnson and Schmidt (1962) pointed out that the high protein genetic trait in wheat is associated with more efficient and complete translocation of nitrogen from the vegetative parts of the plant to the grain. Most of nitrogen in the green plant is in the form of high molecular weight compounds, which must be broken down to lower molecular weight compounds, such as amino acids and small peptides for translocation. Beevers (1968) observed the presence of protease enzymes in pea cotyledons during germination and pointed out that these enzymes

break down the reserve protein into amino acids and other low molecular weight compounds and are translocated to the growing part.

Objectives of the study were:

a) To characterize the protease from the green leaves of the wheat plant,

b) to study the activity of protease during early stages of germination and its influence on the production of free amino acids, tryptophan, indoleacetic acid and growth,

c) to study the seasonal patterns of nitrate reductase and proteases in the green leaves of wheat and their influence on protein production in the grain in selected wheat varieties,

d) to study the effect of added nitrogen on the nitrate reductase and protease activity levels in green leaves and the resulting grain yield and protein production in selected wheat varieties.

CHAPTER II

LITERATURE REVIEW

The atmosphere and soil are possible sources of nitrogen for plants. The atmosphere has vast reserves of elemental nitrogen, with traces of ammonia and other gaseous compounds. Soil contains nitrate, ammonia and organic nitrogen compounds. In most agricultural soils nitrate is the main source of nitrogen for most plants, since nitrogen in the form of ammonia is rapidly converted to nitrates by soil microorganisms (Virtanen and Rautenen, 1951).

While nitrate is the primary form of nitrogen available to the plants growing under field conditions it must be reduced to ammonia before incorporation into keto acids for the synthesis of amino acids and proteins. The plant can and does accumulate nitrate in the vegetative parts at certain stages of morphological development without toxic effects (Hageman et al., 1961).

Miller in 1939 pointed out that nitrogenous substances which are formed in the green leaves of the plant, are translocated from lower leaves to upper leaves and finally to the grain. As most of the nitrogenous compounds in the green leaves are of high molecular weight, they must be converted to low molecular weight nitrogenous compounds

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Abbreviations used throughout this paper: NR-nitrate reductase; NAD-nicotinamide adenine dinucleotide; NADP-nicotinamide adenine dinucleotide phosphate; ATP-adenosine triphosphate; TCA-trichloro acetic acid; PGA-phosphoglyceric acid and 3PGAld-3-phosphoglyceraldehyde.

for translocation. It is generally assumed that hydrolysis of these nitrogenous compounds is affected by proteolytic enzymes.

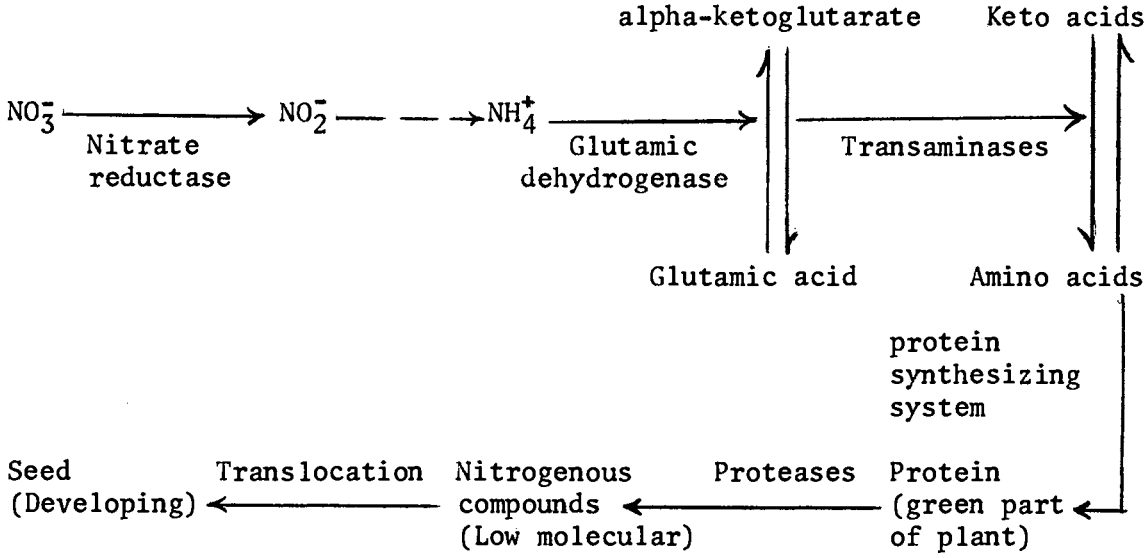

Figure 1. A generalized schematic diagram of nitrogen metabolism in higher plants.

Nitrate Reductase

The enzyme NR was first extracted in a partially purified form from higher plants in 1951 by Evans and Nason, and has not been fully purified or characterized. Many investigators have shown much interest in nitrogen metabolism.

Nicholas and Nason (1955), Evans and Nason (1951) have demonstrated that the NR of soybean leaves is a molybdoflavoprotein enzyme, which catalyses the reduction of nitrate to nitrite using either NADH or NADPH as a cofactor. Subsequent work in 1964 by Beevers et al. showed that NR of many plants has a specific requirement for NADH. Recently, Sims et al., (1968) reported that Lemna minor L. plants exhibited a 20-fold increase in NADPH specific NR when sucrose was added to the culture medium. They indicated the presence of two

enzymes, each with specific cofactor requirement, and the cofactor specificity of NR is determined by the primary energy source. Klepper (1969) observed an appreciable reduction of nitrate to nitrite when PGA, ATP, NADPH and NAD were supplied to the clarified homogenate of corn leaves (3000 X g for 15 min.). He also observed that all four metabolites are essential for the reduction of nitrate. Maximum reduction of nitrite was observed with addition of 3-PGAld and NAD was found to be as effective as NADH alone, whereas, addition of NADPH alone was ineffective. The further reduction of nitrite was formerly thought to involve a series of two electron shifts via hyponitrite and hydroxylamine to ammonia. Evidence presented to date suggests that there are three enzymes NR, nitrite reductase and glutamic dehydrogenase, which may be functioning in a coordinated manner to form a major route for the final assimilation of the nitrogen into organic compounds needed by the plant for growth and development (Fowden 1965).

Evans and Nason in 1953 first showed that young soybean leaves could efficiently utilize either $NADH_2$ or $NADPH_2$, whereas, Beevers et al. (1964) reported that the 15 out of 16 plant species exhibited a specificity to $NADH_2$. Schader et al. (1968) also presented evidence that NR of leaf tissue is $NADH_2$ dependent. Several other natural and artificial compounds have been shown to possess the ability to function as an electron donor for NR. Benzyl viologen was first reported by Hageman et al. in 1962 as an artificial electron donor. Later in 1963 Losado et al. reported ferredoxin, a neutral electron donor would substitute for benzyl viologen in the reduction.

The adaptive nature of NR has been demonstrated both in microorganisms (Nason and Evans, 1963) and in higher plants (Hageman and

Flesher, 1960). However, the induction of enzyme activity does not appear to be completely specific for nitrate as the inducer molecule. Candella et al. (1957) found that cauliflower grown in an ammonium medium, under non-sterile conditions, had appreciable NR activity, whereas, in subsequent studies with plants grown on ammonium sulfate under sterile conditions, NR was extremely low (Afridi and Hewitt, 1964). Studies with higher plant tissue indicated that NR is not repressed by ammonia (Candella et al. 1957), but the induction of NR was found to be approximately proportional to the nitrate level in the tissue (Beevers et al. 1965). Hageman and Flesher (1960) observed an increase in NR activity in corn seedlings with the increase in nitrate content in the nutrient media.

It has been known for some time that the activity of NR in plant leaves exhibits a daily fluctuation (Hageman et al. 1961). Enzyme activity is at its maximum about mid-day and reaches a minimum during the night. The requirement of light for the reduction of nitrate by higher plants was investigated by many researchers. Candella et al. (1957) and Hageman et al. (1960) have shown that cauliflower or corn plants lost NR activity rapidly when placed in the dark. Corn plants lost 50 to 90 percent of their NR activity within 24 and 48 hours respectively after being placed in the dark. The activity of NR is determined by the availability of a cofactor produced by light, which is essential for the reduction of nitrate (Hageman et al. 1961). Burris (1959) suggested that the photoreduction of NADP is the light dependent step in the nitrate reduction. This would indicate an increase in the production of NADPH from morning to mid-day and a decrease thereafter. Darkness, however, did not cause a sharp decrease

in the NR activity, but a slow, continuous decrease. Beevers et al. (1965) demonstrated that light was not an absolute requirement for induction of NR in green leaf tissue as long as sufficient nitrates were present in the induction medium. They indicated that light increased the uptake of nitrate in corn seedlings and this stimulated uptake could be accounted for by the effect of light on induction. In contrast to this Kannagara and Woolhouse (1967) indicated that light and CO_2, in some unknown manner, favors the synthesis of NR in the leaves of Perilla L. Hageman et al. (1961) found maximum NR activity in corn leaves which were fully exposed to the direct sunlight, whereas, artificial shading showed decreased levels. Zieserl et al. (1963) observed a progressive decrease in the leaf NR activity as the plant population increased. They also noted high levels of NR activity and protein content in the top leaves as compared to the bottom leaves.

Temperature is an important factor in nitrogen metabolism. Croy (1967) reported that an increase in temperature caused a decrease in the level of NR in wheat and a similar response was also observed by Younis et al. (1965) in corn seedlings. Travis et al. (1970) studied the effect of temperature in the dark on NR activity levels in barley. They observed a marked decrease in the activity levels of NR in the dark. The loss of NR in the dark was greatly reduced at 3°C, but the loss was rapid, and increased with time when plants were placed in a 24°C regime after 24 hours at 3°C.

Beevers and Hageman (1969) pointed out that nitrate metabolism might be directly associated with the organelles responsible for photosynthesis and respiration. Evans and Nason (1953) originally demonstrated that illuminated chloroplast grana could provide the

electrons for NR. Ritenour et al. in 1963 found that the nitrite reductase is localized in the chloroplast, but not the NR and glutamic acid dehydrogenase. Their data did not preclude NR being bound or associated with the external chloroplast membrane.

Higher levels of NR were found in the leaf extracts when compared to the extracts of petioles and roots. Hageman and Flesher in 1960 observed highest activity levels of NR in the expanded leaves of corn seedlings, whereas, activity decreased as the seedlings aged. This loss was attributed to the proportional increase in the stem tissue in assay. Later Croy (1967) found a high level of activity in the leaves and lower levels in the stem and heads of wheat. Afridi and Hewitt (1964) found that NR activity increased rapidly in cauliflower leaves during the first five weeks of development and remained constant for the next five weeks before declining rapidly. Harper and Paulsen (1967) analyzed individual aerial parts of wheat during the spring, and found that NR activity levels were highest in the blades, with decreasing levels in the sheath, heads and culms respectively, whereas, nitrate contents were highest in the culms with less in the sheath, blades and heads respectively. They also observed that the upper blades and sheaths contained greater NR activity and water soluble protein, while the nitrate concentrations were highest in the lower blades and sheath on a fresh weight basis. All constituents which they measured decreased in all parts of the green plant with maturity. They also observed a significant positive correlation between NR activity and water soluble protein, and between NR activity and nitrate content in crown, blade and sheath tissue.

The literature on the relationship between NR and water soluble

protein is not in agreement. Candella et al. (1957) reported that NR activity was not closely related to the total soluble protein content in cauliflower leaves. In 1962 Toman and Pauli also found no correlation in crown tissue of winter wheat. In 1967 Harper and Paulsen reported a significant correlation between water soluble protein and NR activity in crown tissue of winter wheat. Croy and Hageman (1970) found that the nitrate content of tissue was a major factor in controlling the enzyme activity and NR activity was related to the leaf protein content. In corn leaves Hageman and Flesher (1961) found a significant positive correlation between NR activity and water soluble protein. Zieserl and Hageman (1962) found no correlation between NR activity and water soluble protein. A year later Zieserl et al. (1963) showed that despite no overall correlation, water soluble protein distinctly paralleled NR activity with a 7 to 10 day lag period in corn leaves.

The reports from Zieserl and Hageman (1962), Afridi and Hewitt (1964) and Croy and Hageman (1970) provided ample evidence that NR activity is under genetic control in corn, cauliflower and wheat. Work with cauliflower and corn demonstrated up to five fold differences in the activity levels between genotypes. Hageman et al. in 1963 found that NR activity of F_1 hybrids generally were similar or intermediate to the inbred parents. In 1966 Schrader et al. demonstrated that by proper combination of inbred lines, followed by selection based upon enzyme assay, hybrids could be developed with 'high', 'medium' and 'low' levels of NR activity. Beevers and Hageman (1969) reported on other aspects of a genetic study in which F_1 hybrids obtained by crossing inbreds with 'low' NR levels possessed heterotic levels of

enzyme activity. In 1969 Warner, et al. studied a genetic population of corn and reported that NR activity levels are under control of two-locus system with dominance. Each inbred was homozygous for dominant or partially dominant allele at one locus and homozygous recessive at a second locus. 'B14' a high parent carried a dominant allele at one locus while a 'low' parent 'Oh43' carried a recessive allele at this locus. They observed heterotic levels of enzyme activity in the F_1 hybrid. The data suggest that this heterotic level was the result of an inheritance pattern in which the hybrids possessed intermediate rates of enzyme synthesis and decay, since the two inbreds differed in their rate of enzyme synthesis and in vitro decay. They concluded that the rate of both enzyme synthesis and decay are the factors governing the levels of NR activity in corn.

Influence of amount and time of nitrogen application on the protein production in wheat

The quality and quantity of protein in wheat is of great practical importance. The quality is largely determined by heredity, but the quantity is influenced by conditions of growth and amount of nitrogen available to the plant at different stages of its development. Many studies have been conducted to ascertain the effect of added nitrogen on yield and protein production. Gingrich and Smith (1953) reported that the time of nitrogen application generally had little influence on yield of either wheat or oats. Welch et al. in 1966 reported that a pound of nitrogen applied in the spring increased yield more than a pound of nitrogen applied in the fall. Schiller at al. (1967) were of the opinion that the amount of fertilizer and time of

application produced variation in the protein content of the wheat kernel. Higher fertilizer levels in a series of adjacent plots using the same variety of wheat produced an increase in kernel protein content. Long and Sherbakoff (1951) and Hobbs (1953) were also of the same opinion that the protein yield as well as flour production were influenced by nitrogen fertilization, particularly when applications were made late in the development of the plant.

Influence of foliar application of N on the grain yield and protein content in wheat

Among different methods of N application, foliar application has attracted considerable attention in recent years. Sadaphal and Das in 1966 reported that the foliar application of urea increased the number of kernels per head and 1000 kernel weight. They also observed an increase in the yield when the urea concentration was increased in the foliar application; however, spray concentration above 24 percent of urea was not reported to be beneficial. Sprays applied after blooming were more effective than those applied at heading or blooming for enhancing the accumulation rate of protein in the grain.

Proteases

Proteolytic enzymes which cleave the peptide bonds of protein were discovered in the nineteenth century. Progress came first from an understanding that amino acids are linked in proteins primarily by peptide bonds. By the use of synthetic peptides and their derivatives, convincing demonstrations were brought forward that proteolytic enzymes hydrolyze the peptide bonds (Bergmann and Fruton, 1941). The

recognition of the physiological importance of the proteolytic enzymes was derived in the first instance from the role which such enzymes play in the degradation of dietary proteins to peptides and to free amino acids. It is now recognized, however, that such enzymes are of universal distribution in the living cells, the gastrointestinal tract of animals, plant tissues and microorganisms.

Plant proteases are commonly divided into two classes, the proteinases, which hydrolyze proteins, and peptidases, which hydrolyze the peptides (Bonner and Varner, 1965). This classification can be misleading since proteases will also hydrolyze certain peptides and many peptidases hydrolyze proteins.

Tracey in 1948 reported that the protease content varies in plants growing under different cultural conditions and that caution must therefore be exercised in drawing conclusions from the examination of plants of unknown antecedents and cultural backgrounds. Proteolytic enzymes have been found in both dicotyledonous and monocotyledonous plants (Greenberg, 1955).

Mckee (1962) in his review stated that 'papain', from papaya, which is similar to several other plant proteases, was first studied in 1879 by Wurtz and Bouchut. Several other investigators studied these protease enzymes in seeds of different crops during germination. In order to avoid names, Heinicke and Gortner (1957) called proteases from any member of the Bromeliaceae as 'bromelain'. Other workers such as Irving and Fontain (1945) called peanut proteases 'arachain' and Laufer, Tauber and Davis (1944) referred to soybean protease as 'soyin'. In many other plants no particular name was given to the proteases, or the proteases were referred to as proteases of the particular crop,

among them were wheat (Mounfield, 1936), peas (Beevers, 1968), squash (Penner and Ashton, 1966), cucurbita (Wiely and Ashton, 1967), and rye and barley (Engel and Heins, 1947).

Work on characterizing the nature of leaf protease is limited since the concentration in leaves is low and difficult to purify and isolate. Tracey (1948) studied the activity levels of leaf proteases in different crops and concluded that none of the leaf protease examined approached the protease content of pineapple. Recently the nature of leaf protease in white clover and tobacco was reported by Brady (1961), Kawashima, Fukushima, Imia and Tamakai (1968) respectively.

Proteases during germination

During the initial stages of germination, seed proteins are hydrolyzed into peptides and amino acids. These low molecular nitrogenous compounds are used as substrates for the synthesis of new seedling protein, or are further degraded yielding energy (Webster, 1959; Koller et al. 1962). Bonner and Varner (1965) stated that the maximum rate of hydrolysis of the storage proteins coincides with the maximum rate of growth of the seedlings. They also reported that the cotyledons and endosperms of germinating seedlings not only provide reserve material for the growing embryo, but are also capable of a concurrent *de novo* synthesis of certain enzymes.

Mounfield (1936) observed increased activity levels of proteases (proteinases and dipeptidases) during germination of wheat seeds. His data suggests that very little increase in the activity levels during the first two days of germination, whereas, a ten-fold increase in enzyme activity by day seven after germination was followed by a decline

as germination proceeded. Proskuryakov et al. (1941) stated that wheat grains show a decline in protease content during grain maturation and increased resistance of the grain proteins to proteinase attack, whereas, during germination the situation is reversed. The observation of Wiley and Ashton (1967) on the activity of protease levels in squash seeds during germination showed that the activity increased several fold from relatively low levels at 4 and 24 hours to a high level at two days and gradually decreased to a level approximating the initial value after 7 days. A slight increase in the activity levels of arachain was observed by Irving and Fontain (1945) in peanuts between 48 to 72 hours after seeding and activity decreased as further germination proceeded. Tazakawa and Hirokawat (1956), Laufer et al. (1944) also observed similar patterns of protease activity in soybean seeds during germination. Beevers in 1968 pointed out that most of the rapid increase in the protease activity was observed in the germinating pea seeds after the most rapid depletion of cotyledonary nitrogen. Beevers cited the work of Danielson (1951) indicating that the proteolytic activity was greatest in the extracts of developing seedlings compared to germinating seeds. Shain and Mayer in 1965 observed three different protelytic enzymes in dry and germinating lettuce seeds and concluded that the activity of one enzyme was unchanged during a germination period of 72 hours, while two other types increased in activity as germination proceeded. Doty et al. (1946) found that proteolytic and lypolitic activities in oats and tomato seeds increased greatly with 24 hours of germination.

Distribution of proteases in cereal grain

So far, only Pett (1935) investigated the quantitative distribution of proteases in the germinating wheat grain from a physiological point of view. His data suggest that the hull and endosperm contains a relatively small amount of enzyme and this amount does not change during germination, whereas, activity levels increased greatly in the scutellum and embryo in the first 12 hours of germination, after which they decreased in the embryo but continued to increase in the scutellum for some time. Engel and Heins in 1947 made further studies on the distribution of these enzymes in wheat, rye and barley and reported that the patterns of rye and barley were similar to wheat. Amounts of proteinases and dipeptidases were high in aleurone cells of wheat, rye and barley, but negligible in the endosperm. In the germ they found only a moderate quantity of protease, whereas, dipeptidase activity was high in the epithelial layer of the scutellum. Irving and Fontaine (1945) studied the distribution of these enzymes in peanut seeds and found that the level of enzyme in the cotyledons was higher than in the germ and was absent in the seed coat.

Characterization of proteases

A number of workers have reported different methods for measuring the activity of protease. Since both the substrate and enzyme are proteins, it is difficult to determine optimum conditions of pH, buffer concentration and temperature that are applicable to the enzyme acting on different substrates. Wheat flour was studied extensively by Mounfield (1936), who observed two types of enzymes: endopeptidases,

whose activity was demonstrated by the cleavage of edestin with an optimum pH of 4.1, and dipeptidases, evidenced by the hydrolysis of leucylglycine with an optimum pH of 7.3 to 7.9. There is considerable variation in the pH optima found by different workers for the proteases of malted wheat flour. Mounfield (1936) reported pH optima of 5.1 and 6.4 for hydrolysis of gelatin and gluten respectively, whereas, Miller (1947) reported a pH optima of 3.5 when hemoglobin was used as a substrate.

Traditional substrates for the study of protease action are of animal origin and include casein, gelatin and hemoglobin. An extensive study on properties of proteases by Johnson et al. (1956) pointed out that the fungal and malt wheat flour proteases showed a greater affinity for gluten than for hemoglobin, although the rate of decomposition of the enzyme substrate complex was greater for hemoglobin. They also pointed out that the similarity of the apparent activation energy values for gluten hydrolysis suggests that similar bonds are hydrolyzed by respective enzyme systems. The variation encountered for hydrolysis of hemolobin suggests that different bonds were split by the respective systems. They also reported that pH optima for various enzyme systems were similar, when the same substrate was used for degradation. Johnson (1965) suggested on the basis of his previous findings that malted wheat flour possesses a number of proteases. Beevers (1968) demonstrated the presence of two proteolytic enzymes with pH optima of 5.5 and 7.0 in pea cotyledons. Laufer et al. (1944) reported maximum proteolytic activity in soybean between pH of 6.5 and 7.0 with gelatin and casein as substrates, whereas, Pinsky and Grossman (1969) observed an optimum pH of 5.5 for the same crop when a crude

enzyme preparation was used with soybean protein as the substrate. Shain and Mayer (1965) showed the presence of three proteases in the lettuce seeds with pH optima of 4.8, 5.6 and 6.8. Irving and Fontaine (1945) reported a pH optimum range for purified 'arachain' between 6.0 and 7.0. Bromelain (pineapple protease) was observed to be a complex enzyme consisting of four different types (Heinicke and Gortner, 1957). They also reported two enzymes, active at pH 4.5 and 5.5, which exhibited similar action of splitting certain peptide linkages of gelatin, whereas, two others with optimum pH of 7.0 and 8.5 hydrolyzed hemoglobin and casein.

For the measurement of proteinase activity Ball and Kies (1946) pointed out that the methods used are chosen to fit the needs of the particular problem. They recommended the reduction in the viscosity of gelatin method if the level of protease is low, whereas, hemoglobin if the protease levels were high.

Incubation or digestion temperature has been observed to be one of the most important factors in measuring the activity levels of proteases. Wiley and Ashton (1967) and Beevers (1968) reported an optimum temperature of 40°C for Cucurbita maxima L. and pea seeds respectively, and Wiel, Pinsky and Grossman observed that 50°C was optimum in soybeans.

Factors affecting the nature of proteolytic enzyme activity

Percent germination of seeds are usually taken as a measure of the vitality of seeds. It is reasonable to assume that loss of vitality is accompanied by loss of enzyme activity.

Mounfield (1936) reported that wheat grain stored in stoppered

bottles in the dark at 18°C gradually fell in their power to produce proteinase during germination. Fleming, Johnson and Miller (1960) observed the enzyme activity levels in the freshly harvested wheat and reported the activity did not reach their maximum until after two months of storage. Storage at low temperature (40°F) favored greater production of alpha-amylase and protease.

Many workers recently investigated the effects of hormones on the degradation of seed reserve material during germination. Their results tend to support the idea that the embryo or embryonic axis produces a hormonal stimulus which controls the activation or synthesis of some hydrolyzing enzymes in the storage tissue. Penner and Ashton (1966) reported that the proteolytic activity in the cotyledons (detached from the embryonic axis) of squash seeds was low and that the addition of benzyladenine to the germinating media enchanced the enzyme activity levels to the levels in the intact seedling. Yomo and Iinuma (1962) reported that gibberellin activated the protease in the ungerminated barley endosperm.

Almost all the well known proteases of plant origin are enzymes of the papain type. These enzymes may be reversibly activated and inactivated by many reducing and oxidizing agents, respectively. Among the most suitable activating agents are hydrogen sulfide, cysteine, soluble cyanides and sulfates. Mounfield (1936) observed an increase of 64% in protease activity with the addition of cyanide between 1 to 50 mM concentration in the extraction medium. Brady (1961) observed an increase of 35 to 40 percent in the activity levels of protease with the addition of 200 mM sodium biosulfite and sodium thioglycolate in the crude enzyme preparation. Reducing agents such as

cysteine, glutathione mercaptoethanol and dimercaptoethanol were also observed to increase the activity levels of protease in tobacco leaves by Kawashima et al. (1968).

In recent years, there has been considerable interest in the changes in nitrogen distribution in starving leaves. Oosthuizen and Shedd (1913) observed a considerable decrease in protein content in the leaf during ripening of the tobacco plant and also during curing and fermentation. Tracey (1947) reported on the basis of his preliminary observations that proteases are present in sap and fiber of tobacco leaves. Kawashima et al. (1968) reported that rapid breakdown of protein in tobacco leaves occurs during the process of their curing. They concluded that protein loss from the leaf resulted from protease activity.

Translocation of nitrogen from leaves to the grain

Extensive work has been reported on nitrogen mobilization in the green leaves of wheat plant. Miller (1939) pointed out that the nitrogenous substances which are formed in the green leaves of the plant rises from lower leaves to the upper leaves and finally is translocated into the developing grain. He found that of the total nitrogen in the grain at maturity, 68.1 percent was obtained from the green part of the plant, whereas, 31.9 percent was absorbed directly from the soil. He concluded that greater portion of the nitrogen in the wheat was thus obtained by the plant at a relatively early stage, utilized in the various plant parts, and later translocated to the developing grain. Seth et al. in 1960 observed no differences in protein content among 'high' and 'low' protein wheat varieties until the heading stage.

However, at milk stage and continuing to maturity, the vegetative plant parts of 'high' protein varieties decreased in protein content more rapidly than 'low' protein wheats. 'High' protein wheat varieties also exhibited more rapid increase in protein percent in the seed than 'low' protein wheat varieties. It was concluded from those observations that differences in protein content were associated with differences in rate of protein degradation in the plant or protein synthesis in the developing kernel. The study conducted by Haunold et al. in 1962 on variation in protein content of grain in 4 wheat varieties agrees closely with that of Seth et al. (1962) and Johnson et al. (1963). They stated that the protein in the grain of wheat results from the translocation of nitrogenous compounds from other parts of the wheat plant. The level of nitrogen in the wheat plant, in turn, is affected by the availability of nitrogen in the soil in which the wheat is grown. Johnson et al. (1963), in determining the agronomic and quality characteristics of 'high' protein F_2-derived families from a soft red winter X hard red winter wheat cross, found that some families produced additional protein in the grain without any decrease in the total grain production. They also presented evidence that expression of 'high' protein characteristics in these families was not dependent on high soil nitrogen, but could be detectable at low nitrogen levels. In 1968 Johnson, Schmidt and Mattern studied the physiological effect of these 'high' protein wheat parent genes (Atlas 66). They found that the 'high' protein genetic trait in wheat is not associated with differential nitrogen uptake or nitrogen accumulation in plant, but due to more efficient and complete translocation of nitrogen from the plant to the grain. They also observed that higher protein in wheat over a

range of fertility levels could be expected from the varieties which possess the genes from Atlas 66.

CHAPTER III

MATERIALS AND METHODS

Characterization of Protease

Plant material used in characterizing the proteases were grown in a controlled environmental chamber during the summer of 1969. Wheat seeds of NB65317 were sown in plastic trays using perlite (Zonolite Division, W. R. Grace and Company, Cambridge, Massachusetts) as a supporting media for growth with 16 hour days and 75-65 F (24-18°C) day-night temperatures. The plants were subirrigated daily with modified Hoagland's No. 1 nutrient solution. Germinated seedlings were collected after 7 days of seeding and the enzyme was characterized for optimum pH, enzyme concentration, substrate concentration, incubation temperature and period of incubation. The analytical procedures are discussed in a later part of this chapter.

Developmental Study

This study was also conducted in a controlled environmental chamber using NB65317 and Triumph 64. Similar environmental conditions were provided for growth as described above. Adequate moisture was maintained at all times, but no nutrients were given in order to prevent any nutrient-genetic interaction for protease and protein degradation. Total plant samples were taken after seeding on days 3, 5, 7, 9 and 11 respectively. Sampling ceased when senescence became

obvious. Samples were analyzed for protease, water soluble protein, total amino acids, tryptophan and IAA. On each sampling date total plant height was recorded.

Field Studies

Field nurseries were grown on the Agronomy Research Station of Oklahoma State University at Stillwater. The experiment was conducted in 1968-69 (Study 1) and was repeated in 1969-70 (Study 2) with slight modifications.

Four wheat varieties were used in both the studies: Nebraska Selections-65317 and 65679, Purdue Selection-B4930 and Triumph 64. Nebraska Selections are crosses of Atlas 66 (a soft winter wheat) with Comanche (hard winter wheat) made in 1953 at Lincoln, Nebraska. In 1965 the Nebraska Selections were released and obtained by Oklahoma State University. Purdue Selection B4930 was obtained from Purdue University, West Lafayette, Indiana and is also an Atlas parentage genotype. Nebraska and Purdue Selections are high in grain protein content. Triumph 64 is a variety widely grown in Oklahoma and has low protein content when compared to other varieties used in this study. It is very popular due to its early maturity. Seed material was grown at Stillwater for two years before it was used for these studies.

Cultural Practices

Study 1. This trial was conducted on a Kirkland silt loam soil. A uniform basal application of 18-20-0/A was given to all the plots as a preplant application.

Plot size consisted of 4 rows each 10 feet long with a 12 inch

spacing between rows and arranged in a randomized complete block design with four replications. The varieties used in this trial were planted on September 28, 1968. On February 22, 1969 an application of 60 lb./A. of actual nitrogen (as NH_4NO_3) were applied uniformly to all plots.

Study 2. This trial was conducted on a Port silt loam soil. Experimental design, wheat varieties, plot size and basal application of fertilizer were similar to those of Study 1. The trial was planted on October 28, 1969. Two fertility levels of 60 and 120 lb. of actual nitrogen/A. (as NH_4NO_3) were applied. Each fertility level was applied on April 5, 1970 in two replications selected at random. Plant samples were collected in the spring for the measurements of enzyme levels.

Sampling Procedures

Fresh leaf blade samples were collected at random from each experimental unit, except from the two center rows, which were used to determine the grain yield and protein percent. Plant samples were placed in plastic bag immediately after harvest and were covered with ice, to prevent any loss of enzyme activity. Leaf samples in Study 1 were collected in the fall and spring on the dates of: November 20, December 3 and 13, February 11 and 27, March 13 and 27, April 3, 10 and 22. On all the above dates the leaf samples from all positions of the plant were pooled and analyzed for NR, protease, water soluble protein and protein percent. Leaf samples collected in May were analyzed separately as bottom, middle and top leaves on dates (May 1, 8, 14 and 26) for protease and water soluble protein. In Study 2

leaf samples were collected and pooled on April 7, 14, 21 and 28; May 5 and 12 for analysis of NR, proteases, water soluble protein, percent protein and nitrate content. Later leaf blade samples were analyzed separately as middle and top leaves on May 21 and only top leaves on May 28 for proteases, water soluble protein and percent protein.

Analytical Procedures

In the laboratory crude nitrate reductase enzyme preparations were made as previously described by Croy and Hageman (1970), using 6 ml of grinding media containing 50 mM potassium phosphate, 10 mM cysteine, 35 mM EDTA, pH adjusted to 9.0 with KOH, for each gram of finely cut fresh leaf tissue. Leaf samples were ground using a Omnimix homogenizer at full speed for 2 minutes. The plant extracts were filtered through cheese cloth, centrifuged at 13,000 rpm (20,850 X g) for 15 minutes at 0°C, with the supernatant fluid used for the enzyme assay. The assay procedure used was that of Croy and Hageman (1970).

Crude enzyme preparations for proteases were made in a similar manner to that of NR with the slight modification that pH was adjusted to 7.0. Assay procedures carried out as described by Kuo and Yang (1966) with certain modifications. Assay tubes containing 1.0 ml of freshly prepared 1% hemoglobin solution in citrate-phosphate buffer consisting of 15.4 mM citric acid and 16.5 mM sodium phosphate for protease active at pH 4.0 and 3.3 mM citric acid and 21.8 mM sodium phosphate for protease active at pH 7.0. Enzyme extract of 0.1 ml was added to duplicate tubes and 1.1 ml of 5 percent TCA solution was immediately added to one tube (blank) to precipitate the enzyme and

hemoglobin. Assay tubes along with blanks were incubated at 40°C for two hours and then the reaction in the assay tube was stopped with 1.1 ml of 5 percent TCA. Samples were centrifuged at 2000 rpm (1000 X g) for 15 minutes to sediment the undigested hemoglobin. The supernatant fraction was collected and analyzed by the Lowry method (Lowry et al. 1951) for digested nitrogenous substances, as a measure of the protease activity using bovine serum albumin as a reference.

Water soluble protein content of the crude enzyme extract was estimated by the Lowry et al. (1951) procedure, using 5 percent TCA precipitable material. Nitrate contents were estimated by the method of Woolley et al. (1960). Total amino acids were determined as amino nitrogen using the ninhydrin test of Yemm and Cocking (1955) with isoleucine as a reference. Percent protein was estimated by the micro-Kjeldahl procedure. Tryptophan content was measured by fractioning 5 ml of crude enzyme through the Sephadex G-25 column. The elution fractions (5 ml each) from tube number 36 to 38 exhibited UV absorption typical of tryptophan. These fractions were pooled and along with standard tryptophan, were separated on thin layer chromatography to demonstrate the presence of tryptophan. The Rf values of the fractions exactly matched those of standard tryptophan. No other amino acids were observed on the thin layer plates. Tryptophan content of the pooled fractions was then estimated with the ninhydrin method using tryptophan as a reference for quantitation. Gordon's procedure (1955) was used in estimating the IAA content. Fresh leaf samples were freeze dried and used for the extraction of IAA. Later the extracted sample was chromatographed in duplicate to separate IAA. One chromatogram was sprayed with an indicator to locate the IAA and the

unsprayed chromatogram was then cut into thin strips of equal length. The quantity of auxin on the chromatogram was determined by bioassay using the growth response of oat coleoptiles.

CHAPTER IV

RESULTS AND DISCUSSION

Verification of Assay Conditions for Protease

The results of assay conditions for protease activity are shown in Figures 2 and 3 respectively. The variables tested were: (a) pH, (b) enzyme concentration, (c) substrate concentration, (d) incubation temperature and (e) period of incubation. All the assay conditions were carried out in a citrate-phosphate buffer as described in the methods and materials.

The effect of pH on the protease activity would indicate that there are two proteases present, one active at pH 4.0 and the other at pH 7.0. Similar results on the activity levels at pH 5.0 and 7.5 were observed in germinating wheat seeds by Pet[+] in 1960, which he termed as protease (pH 5.0) and dipeptidase (pH 7.5). Protease which is active at pH 4.0, cleaves the protein into lower molecular protein compounds as evidenced by the peak of water soluble protein; whereas, protease active at pH 7.0 cleaves the protein or peptides into amino acids.

A linear relationship existed between the amount of enzyme and the extent of protein hydrolysis or activity occurring over the amount of enzyme ranging from 0.025 to 0.3 ml.

Substrate concentration ranged from 0.05 to 1.4 percent indicated a maximum activity for protease (pH 4.0) over 0.3 percent, whereas,

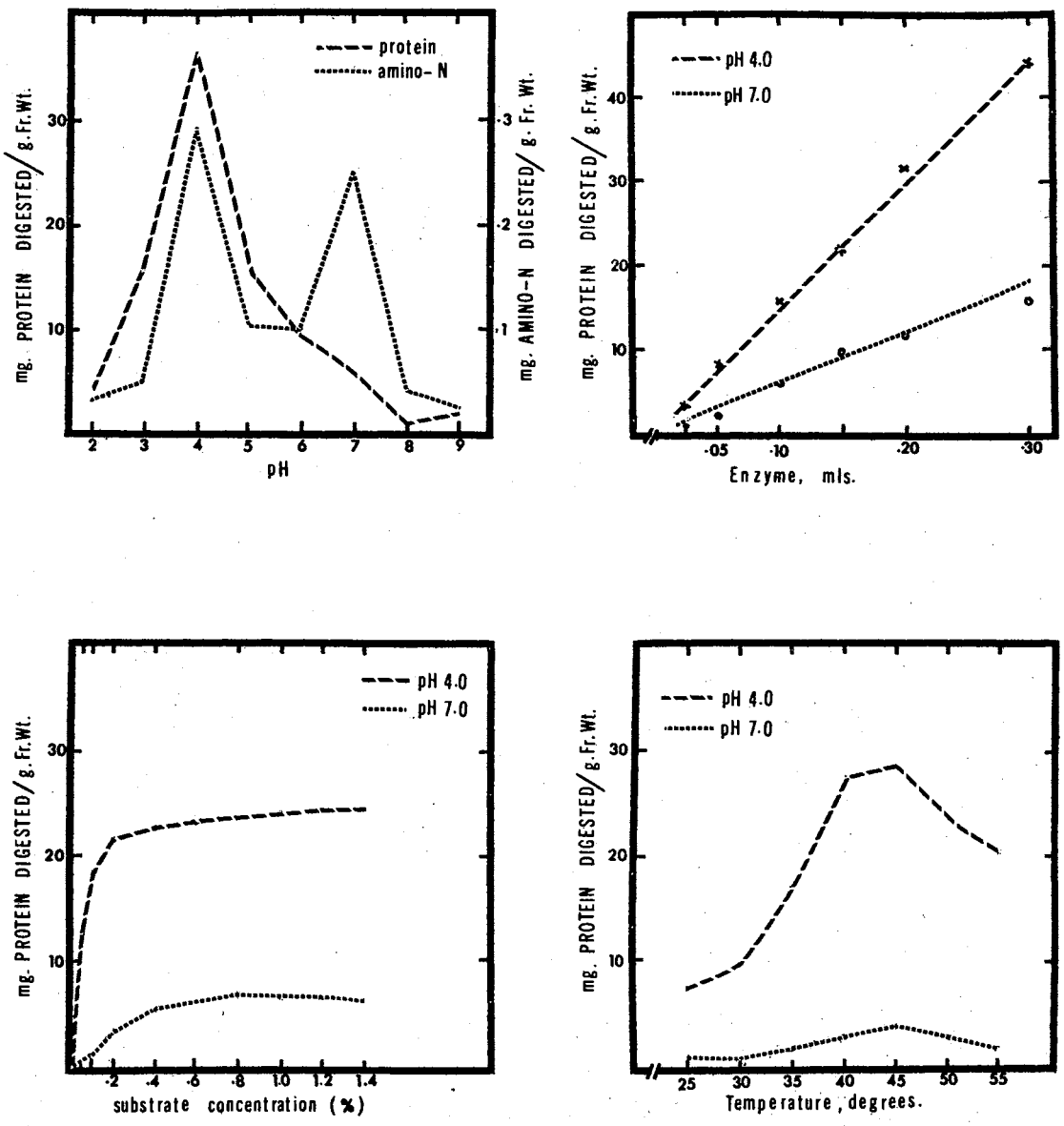

Figure 2. The effect of pH, enzyme concentration, substrate concentration and temperature on the activity of protease enzyme of wheat leaves.

Figure 2. The effect of pH, enzyme concentration, substrate concentration and temperature on the activity of protease enzyme of wet leaves.

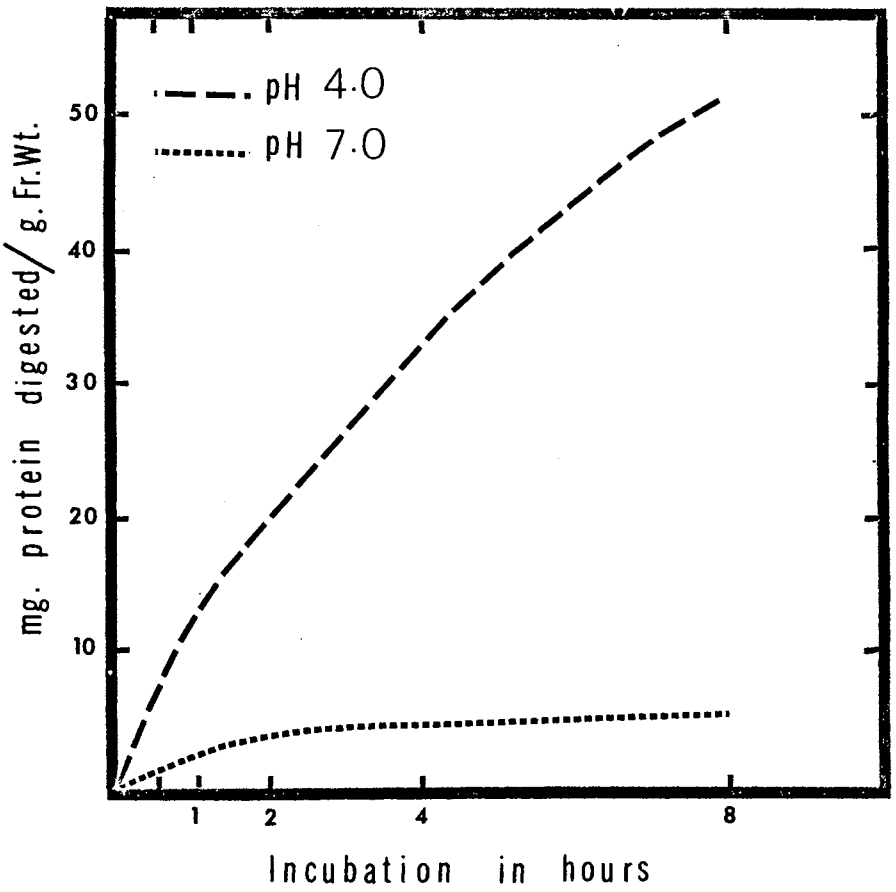

Figure 3. Effect of period of incubation on the activity of protease enzymes of wheat leaves.

protease (pH 7.0) over 0.6 percent. The lower substrate concentration levels exhibited an approximate linear relationship. In all experiments enzyme concentration was 0.1 ml.

Incubation temperatures ranging from 25 to 55°C were used to determine the optimum temperature for the maximum protease activity. Increased activity levels were observed up to 40°C, remained essentially constant from 40 to 45°C and then declined. The optimum temperature appears to be between 40 to 45°C.

Periods of incubation (30 minutes to 8 hours) for both protease enzymes indicated that the rate of hydrolysis of protein was rapid up

to 1 hour period, then the rate of hydrolysis declined with further incubation.

Developmental Study

Protease levels in Nebraska Selection 65317 and Triumph 64, and their relationship to the production of amino acids, tryptophan, and IAA during germination and early growth

Protease activity of the germinating wheat seedlings is shown in Figure 4. The activity levels were measured on days 3, 5, 7, 9 and 11 after seeding. The protease levels were greater in NB65317 than Triumph 64. The NB65317 exhibited a gradual increase in the protease activity and reached its highest activity on the 5th day after planting. As germination proceeded the activity levels declined. The protease activity levels for Triumph 64 declined throughout the sample period. The seed was sufficiently hard so that an enzyme extract could not be obtained before the 3rd day of germination; therefore, we were unable to ascertain the protease levels during this time interval. The peak for Triumph 64 probably occurred during this period.

The two wheat varieties had similar patterns of water soluble protein during the sampling period. In both varieties (Figure 4) the water soluble protein peaks occurred on the 5th day after germination and then declined. NB65317 showed higher water soluble protein content than Triumph 64.

The free amino acids content as shown in Figure 4 increased greatly in the NB65317 from 3rd to 5th day, then gradually declined.

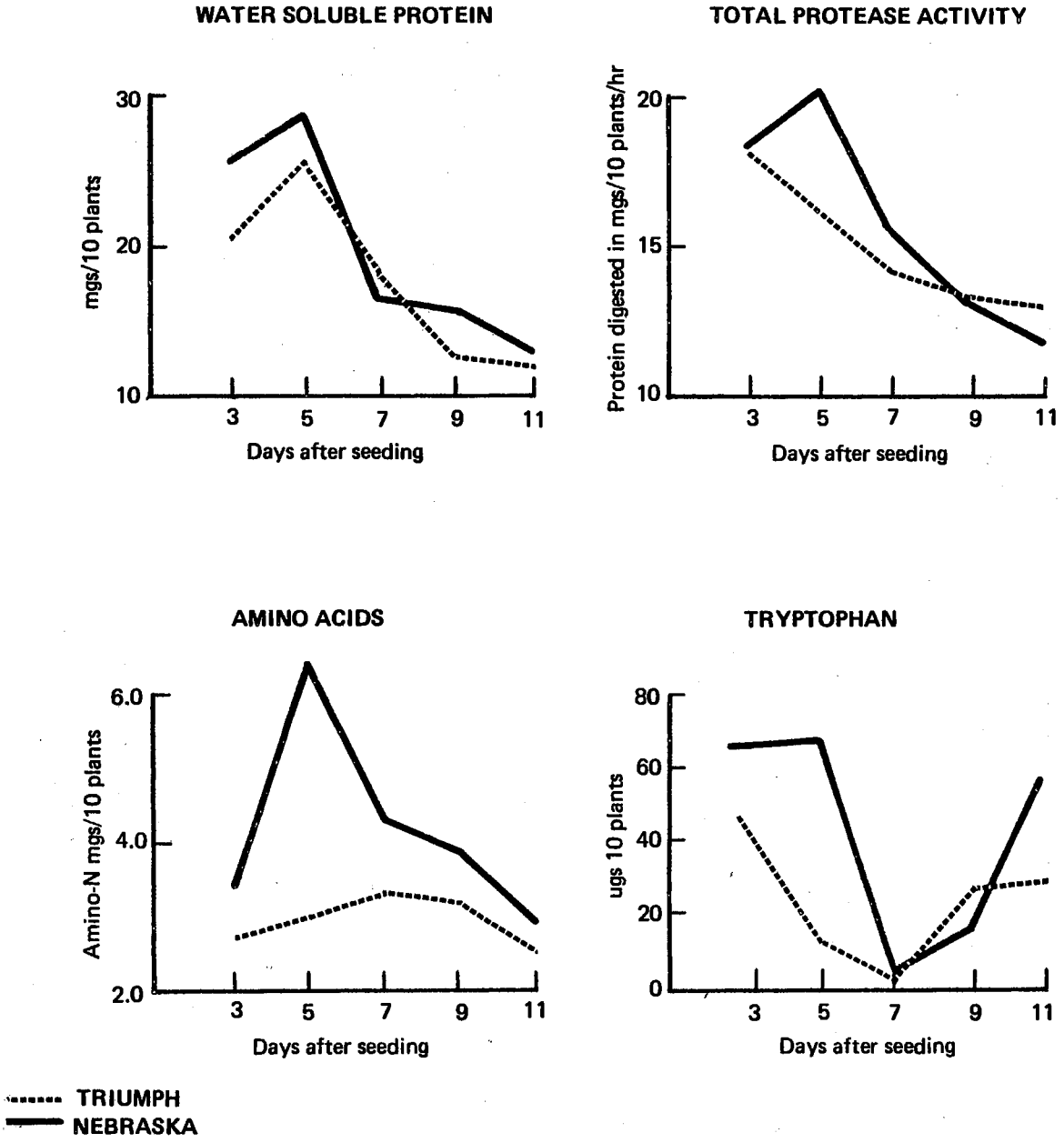

Figure 4. Leaf water soluble protein, protease, amino acids and tryptophan contents for Nebraska and Triumph wheat varieties during early growth.

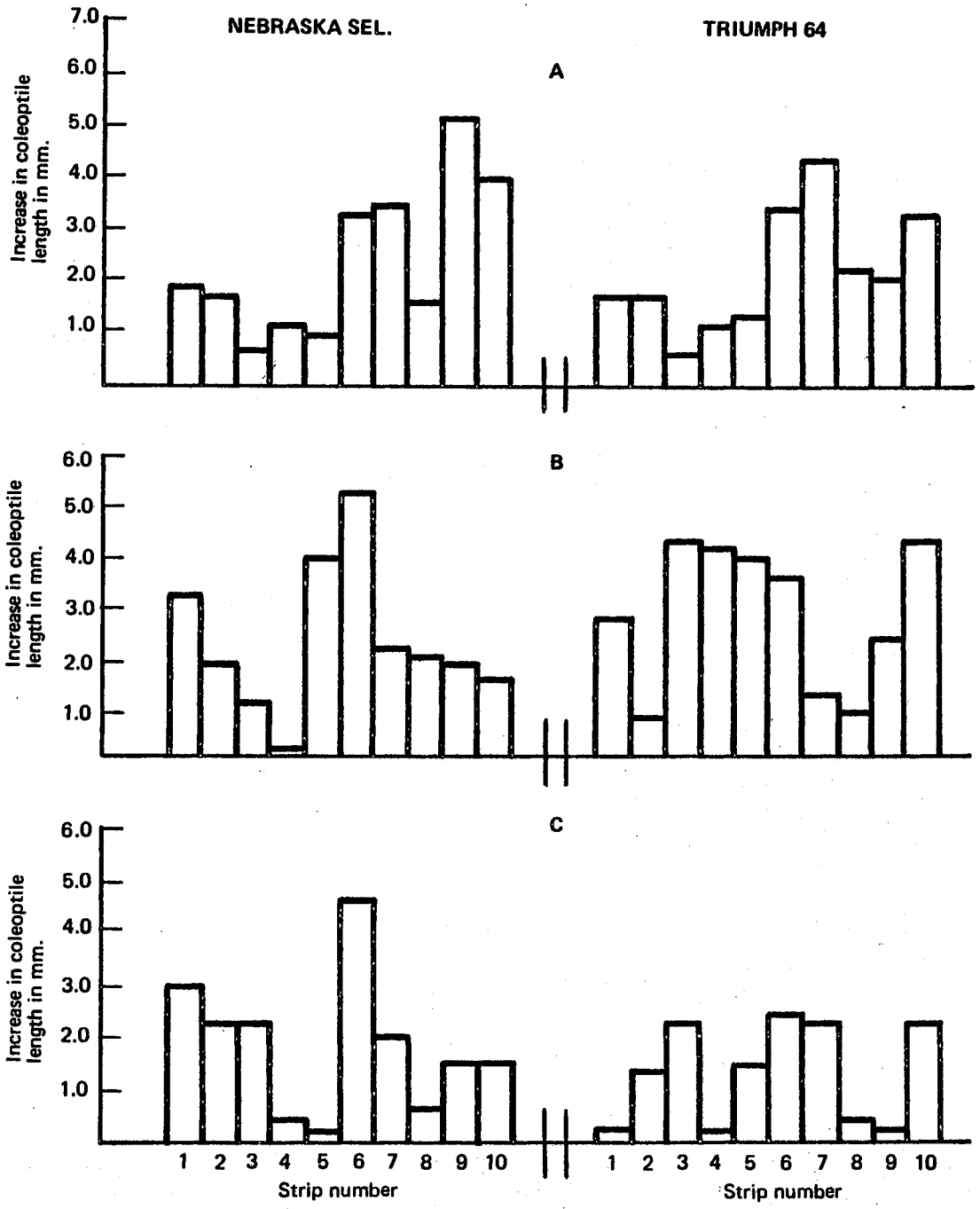

Figure 5. Growth promotive substances on the chromatogram strips as evidenced by the coleoptile elongation during early growth of wheat. A-3 days, B-7 days, and C-11 days after seeding.

This increase correlated with the high protease activity, whereas, amino acid content for Triumph 64 did not correlate with protease activity.

The tryptophan content declined as germination proceeded up to day 7 (Figure 4). On day 7 both the varieties had their lowest content of tryptophan and increased as the growth proceeded further. This decrease in tryptophan content coincided with the peak production of IAA in NB65317. The tryptophan increase in the later germination period probably is the result of plant senescence.

The known IAA and plant samples exhibited a growth response on strip 6 and 7 of the chromatogram. Other growth promotive substances were also observed on strips 3, 4, 5, 9 and 10 in a manner similar to that of Nitsch (1956). IAA for NB65317 (strips 6 and 7) increased gradually as the germination proceeded until day 7 (Figure 5) and then declined. NB65317 exhibited a total of 22 percent greater production of IAA as evidenced by growth of coleoptiles than Triumph 64 throughout the sampling period.

NB65317 exhibited 10 percent greater height than Triumph 64 (Table I). The rate of increase in height in both the varieties was quite rapid until day 7 with no growth after day 9.

Increase in protease activity in NB65317 was followed by an increase in water soluble protein and amino acid contents. The growth patterns between the two varieties would also indicate that the increase in growth for NB65317 could be the result of a greater increase in protease activity, amino acid and IAA production than that of Triumph 64. Plant heights were much greater for NB65317 even though the size of the seed was similar for the two varieties. The major difference

between these seeds was the protein percent and this difference apparently could have promoted an increase in growth by NB65317.

TABLE I

AVERAGE PLANT HEIGHTS FOR NB65317 AND TRIUMPH 64 DURING GERMINATION AND EARLY GROWTH

Variety	Days after planting 3	5	7	9	11
	Plant height in cm				
NB65317	14.9	25.7	37.1	43.3	41.0
Triumph 64	11.8	23.4	35.6	39.0	38.1

The tryptophan content and IAA patterns evidenced inverse relationships. The tryptophan content was low on day 7, perhaps as a result of reduced amino acid content and also as a result of a high rate of synthesis of IAA. There was an inverse relationship in the level of tryptophan and IAA suggesting that this precursor was utilized in the synthesis of IAA and this also could be a contributing factor to a greater growth in the NB65317.

Seasonal Patterns of Field-grown Plants

Seasonal patterns of nitrate reductase, protease and other nitrogenous components before flag leaf stage in Study 1 (1968-69)

The seasonal patterns of NR, protease, water soluble protein and protein percent of four wheat varieties; Nebraska Selections: 65317, 65679, Purdue Selection B4930 and Triumph 64 are graphically presented

in Figures 6 and 7. Table II presents the analysis of variance pooled over all sample dates before the flag leaf stage. Of significant interest in the table are: (a) NR activity levels among varieties and among dates were found to be highly significant, (b) a significant interaction existed between variety X date, (c) NR levels were high in the fall and (d) higher levels of NR were observed early in the spring and then declined as the season progressed. In general, high grain-protein wheats possessed higher levels of NR activity throughout the sampling period. All the high grain-protein varieties exhibited an increasing trend in NR activity levels after the nitrogen application on February 22. Triumph 64 reached its maximum NR levels on February 27; whereas, the high grain-protein wheats reached their highest activity levels on March 13 and then declined.

There were no differences in protease activity nor water soluble protein content among varieties nor any variety X date interaction; however, differences among dates were observed. All varieties exhibited gradual increases in protease activity levels until the protease reached its maximum activity levels on April 10 and then declined. High levels of water soluble protein content were observed in the spring growth. Maximum amounts of water soluble protein were observed in all varieties on two sampling dates, March 6 (probably as the result of high NR levels) and April 10 (probably as the result of high protease levels).

Kjeldahl protein percent in the leaf before flag leaf stage is shown in Figure 7. No significant differences were observed among varieties or dates. In general all varieties exhibited a steady and significant decline in Kjeldahl protein in plant tissue.

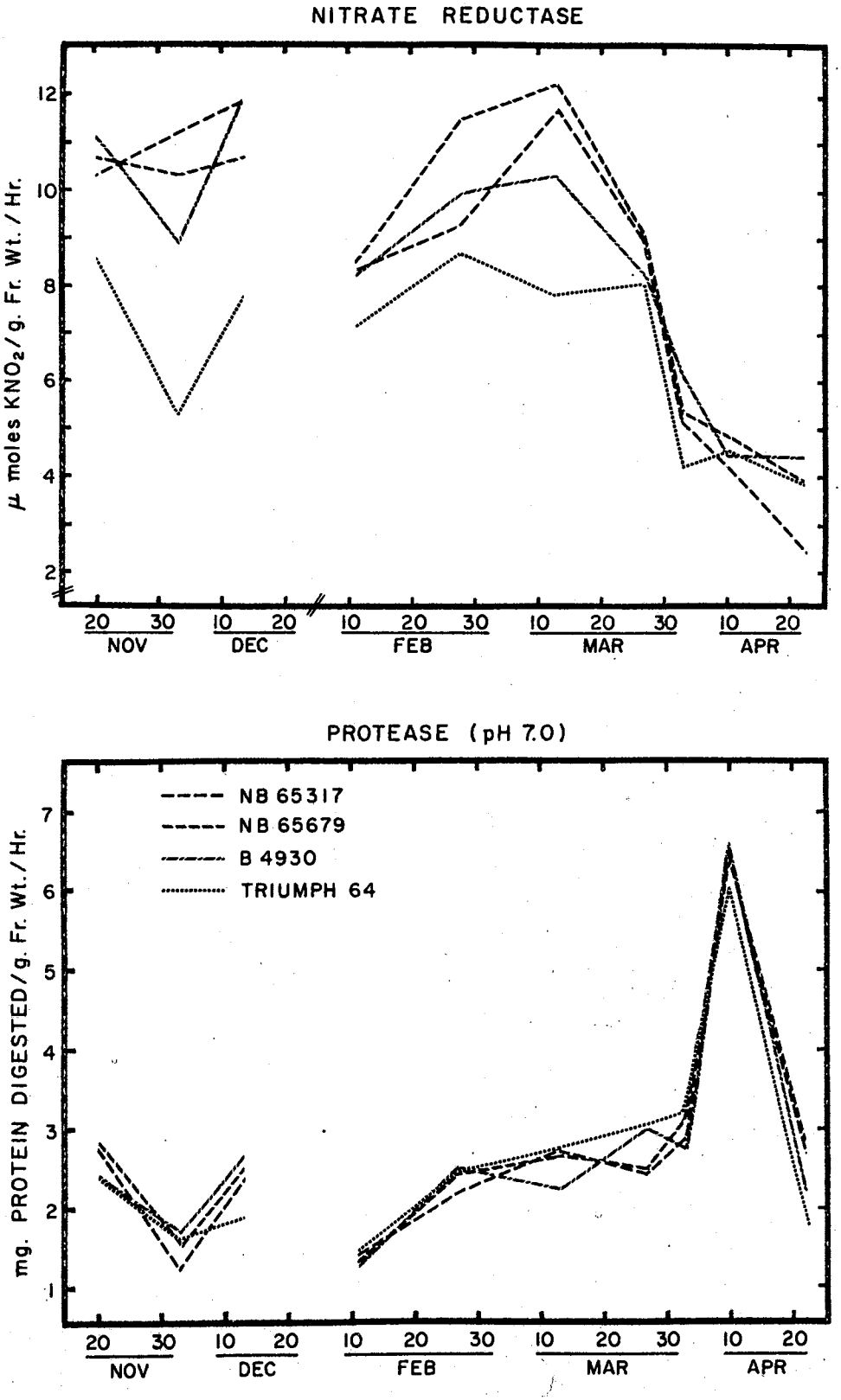

Figure 6. Patterns of leaf NR, protease before the flag leaf stage for wheat. Supplemental nitrogen (60 lb. of N/A.) was applied February 22, 1969.

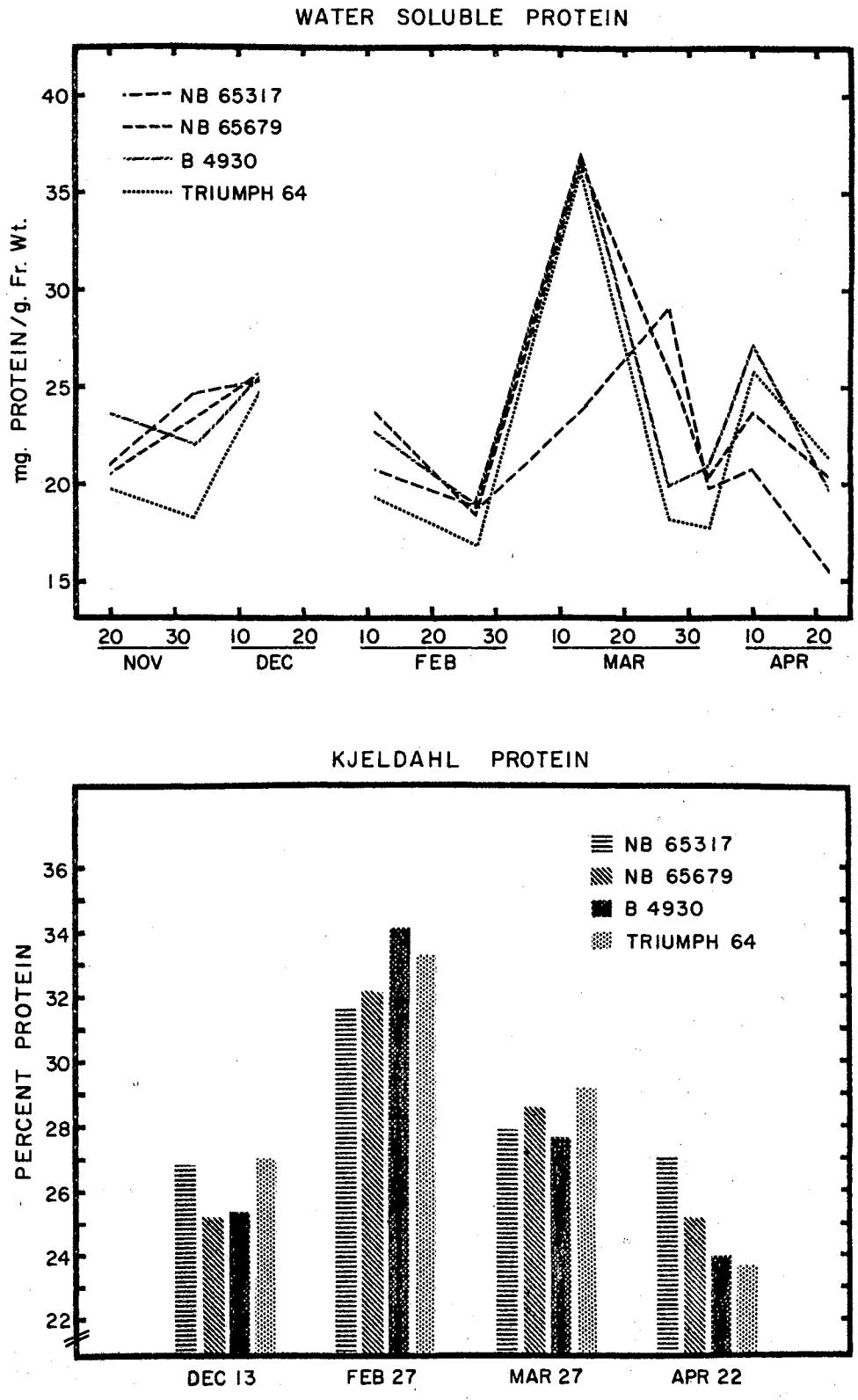

Figure 7. Patterns of leaf water soluble protein and percent Kjeldahl protein before the flag leaf stage for wheat. Supplemental nitrogen (60 lb. of N/A.) was applied February 22, 1969.

TABLE II

ANALYSIS OF VARIANCE FOR LEAF NR, PROTEASE, WATER SOLUBLE PROTEIN BEFORE FLAG LEAF STAGE IN 4 WHEAT VARIETIES (1968-69)

Source	DF	NR	Protease	Water soluble Protein
Total	159	9.2663	1.9402	44.6798
Replications (R)	3	0.2783	0.1047	70.6012
Varieties (V)	3	37.1155*	0.1770NS	49.0892NS
Error (a)	9	4.4548	0.1494	15.1132
Dates (D)	9	105.0077**	30.2726**	308.9371**
V X D	27	4.4784**	0.3160NS	40.0817NS
Error (b)	108	2.3619	0.2341	25.4450

NS not significant
*significant at 0.05 level
**significant at 0.01 level

After flag leaf stage

Only proteolytic activity and water soluble protein contents were determined in leaves from top, middle and bottom parts of the plant after the flag leaf stage (Table III). The analysis of variance are shown in Table IV. Nitrate reductase measurements were not made since activity levels were very low. The following points are significant: (a) protease activity among varieties was not different; however, differences among leaf position and dates were observed to be significant at 0.01 significance level. All the varieties were significantly higher in protease levels on May 8, and later declined as physiological maturity approached. (b) The other differences found to be significant were the interactions: variety X date, and variety X date X leaf position. With regards to variety X date interaction, Triumph 64 was high on early dates, whereas other varieties were high on later dates. (c) Protease activity levels increased in all leaves up to May 8, and then declined. In general it was observed that the decline in protease activity was slower in the top leaves than the middle leaves of the plant; whereas, a rapid decline was found in the bottom leaves. On the last date of sampling (May 26) the high grain-protein wheats still had higher activity levels in the top leaves than did Triumph 64. At this time both bottom and middle leaves were nearly dead and had very low enzyme levels. Sampling ceased since all leaves were senescing.

Water soluble protein contents were different (0.01 significance level) for varieties, position of leaves, dates and interaction of variety X date; leaf position X date; and variety X date X leaf position. All varieties exhibited gradual declines in water soluble

TABLE III

MEANS OF LEAF PROTEASE ACTIVITY AND WATER SOLUBLE PROTEIN AFTER THE FLAG LEAF STAGE OF 4 WHEAT VARIETIES (1968-69)

Varieties	Position of leaves	Protease mgs. protein digested/g. Fr. Wt.				Water soluble protein mgs/g. Fr. Wt.			
		May 1	May 8	May 14	May 26	May 1	May 8	May 14	May 26
NB65317	Top	3.06	8.86	5.42	3.41	21.41	22.72	27.21	11.18
	Middle	3.08	9.91	5.36	0.96	18.64	14.11	14.72	3.43
	Bottom	3.84	6.97	2.19	0.49	10.18	6.17	4.26	2.76
NB65679	Top	2.67	9.09	4.75	3.46	21.85	25.80	29.73	11.86
	Middle	2.61	9.27	5.64	0.46	20.74	15.41	18.41	3.23
	Bottom	3.66	7.87	3.74	0.00	10.78	8.08	6.19	2.46
B4930	Top	2.30	9.07	4.91	4.73	22.63	26.06	32.06	17.64
	Middle	2.61	8.15	4.95	2.80	23.02	18.80	20.77	4.47
	Bottom	3.19	8.25	4.18	0.24	10.90	8.74	8.34	2.01
Triumph 64	Top	2.81	10.47	5.21	1.19	22.61	23.78	21.93	3.74
	Middle	3.74	11.58	5.78	0.52	18.89	12.95	8.05	2.23
	Bottom	3.72	8.30	1.24	0.12	8.18	8.18	1.97	2.23

TABLE IV

ANALYSIS OF VARIANCE FOR LEAF PROTEASE AND WATER SOLUBLE PROTEIN OF 4 WHEAT VARIETIES AFTER FLAG LEAF STAGE (1968-69)

Source	DF	Protease	Water soluble protein
TOTAL	191	10.019	79.410
Replications (R)	3	0.272	21.464
Varieties (V)	3	0.318^{NS}	249.445**
Leaf position (P)	2	38.823**	3729.552**
V X P	6	1.929^{NS}	9.249^{NS}
Error (b)	24	0.909	6.077
Dates (D)	3	494.395**	1431.498**
V X D	9	5.677**	24.859**
P X D	6	16.412**	199.597**
V X P X D	18	2.306**	15.461**
Error (c)	108	0.989	4.461

^{NS}not significant
*significant at 0.05 level
**significant at 0.01 level

protein contents in the bottom leaves from May 1 to May 26. The decline in water soluble protein content was slow in the middle leaves of high protein wheats up to May 14 and later declined rapidly; whereas, Triumph 64 showed a rapid decline from May 8. Top leaves of the high protein wheats increased in water soluble protein until May 14, and later declined; whereas, Triumph 64 did not exhibit any increase but declined in a manner similar to the other varieties.

Inspection of the above data for protease and water soluble protein in relation to plant leaf position in the month of May, suggests that the protease activity in the leaves near maturity peaked and declined in sequence starting with the bottom leaves, proceeding to the middle, and then the top as these leaves began to senesce. The water soluble protein levels increased in the respective leaves in response to the protease activity levels, which were present, then declined as translocation of the nitrogenous constituents to a higher plant part (middle or top leaves or the seeds) occurred. The high grain-protein wheats had higher soluble protein in the top leaves than did Triumph 64 even on the last date of sampling. Higher water soluble protein contents in the upper leaves of high grain protein varieties could be the result of hydrolysis of leaf protein by proteases. Triumph 64 is an early maturing variety and this could explain the shorter period of protease activity and reduced water soluble protein content.

Relationship of grain and straw protein production to NR and protease activities

Table V presents the averages of grain and straw yields, protein

TABLE V

GRAIN AND STRAW YIELD, PERCENT PROTEIN AND PROTEIN PRODUCTION PER ACRE OF 4 WHEAT VARIETIES (1968-69)

	Grain			Straw		
Variety	Yield Bu/A.	Percent protein	Protein lbs/A.	Yield lbs/A.	Percent protein	Protein lbs/A.
NB65317	37.1	17.05	366.9	5548	3.34	185.3
NB65679	31.9	17.02	323.2	5349	4.05	216.3
B4930	37.1	17.50	388.5	5143	4.06	208.8
Triumph 64	39.3	14.85	322.7	4554	3.82	173.9

percent and protein in pounds per acre. No significant differences were observed in grain yield, straw nor straw-protein among varieties, however, a significant difference was observed in the grain-protein percent among varieties. Protein percent in the grain of all the high grain-protein wheats was higher than Triumph 64. Protein production per acre was also higher in NB65317 and B4930 than in Triumph 64 and NB65679. Straw-protein percent was higher in NB65679 and B4930 than the other varieties. This could be due to lack of translocation to the developing seed as evidenced by the presence of a higher amount of water soluble protein on the last date of sampling in the top leaves.

These data suggest that there is a relationship between the activity levels of NR and proteases (especially the duration of protease after flag leaf stage) to that of grain-protein content. All the high grain-protein wheats exhibited higher levels of NR resulting in increased protein percentage and protein production per acre (except NB65679). A similar response of NR activity and their influence on the increase protein production in grain was observed by Croy and Hageman (1970).

Seasonal patterns of NR, proteases and other nitrogenous components in study 2 (1969-70), before flowering stage

Analysis of variances for NR, proteases, water soluble protein, protein percent and nitrate contents for four wheat varieties before flowering stage are presented in Table VI.

The seasonal patterns of NR, and protease activities are graphically presented in Figure 8. As was pointed out in the previous

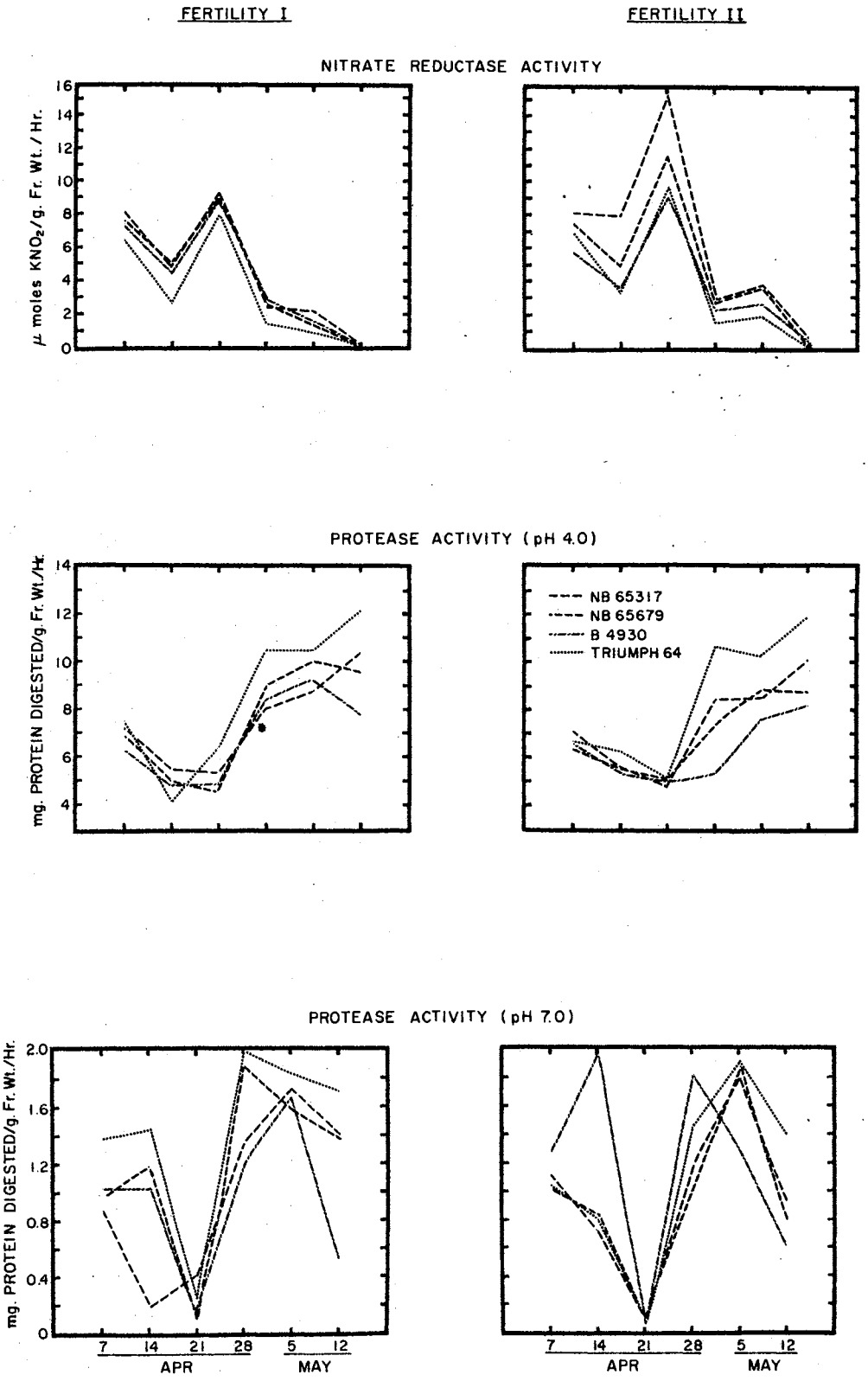

Figure 8. Patterns of leaf NR, and proteases (pH 4.0 and 7.0) before the flowering stage for wheat. Fertility I (60 lb.) and Fertility II (120 lb.) N/A was applied April 5, 1970.

TABLE VI

ANALYSIS OF VARIANCE FOR LEAF NR, PROTEASE (pH 4.0 AND 7.0), WATER SOLUBLE PROTEIN, PERCENT PROTEIN AND NITRATE CONTENT OF 4 WHEAT VARIETIES (BEFORE FLOWERING), IN STUDY 2, 1969-70

Source	DF	NR	Protease (4.0)	Protease (7.0)	W.S. protein	Percent protein	Nitrates X 10^3
Total	95	13.4	5.0	0.5	31.7	7.0	204
Replications (R)	1	0.4	0.1	0.1	25.6	20.1	80
Fertility (F)	1	15.3^{NS}	2.6^{NS}	0.1^{NS}	1.1^{NS}	0.8^{NS}	15^{NS}
Error (a)	1	3.7	0.7	0.7	5.5	11.3	131
Variety (V)	3	15.9**	14.9**	0.4^{NS}	34.9^{NS}	4.4^{NS}	338**
F X V	3	4.9**	0.4^{NS}	0.4^{NS}	27.1^{NS}	2.6^{NS}	49^{NS}
Error (b)	6	0.4	0.7	0.3	7.7	4.1	18
Dates (D)	5	219.8**	66.0**	4.4**	464.2**	89.3**	231
F X D	5	4.1**	1.8^{NS}	0.1^{NS}	5.4^{NS}	4.9*	66^{NS}
V X D	15	1.5*	2.2**	0.3^{NS}	8.9^{NS}	1.3^{NS}	116^{NS}
F X V X D	15	1.1^{NS}	0.9^{NS}	0.2^{NS}	3.3^{NS}	1.4^{NS}	18^{NS}
Error (c)	40	0.6	0.8	0.3	5.3	1.8	95

^{NS}not significant

*significant at 0.05 level

**significant at 0.01 level

study, no detailed observations were taken at different developmental stages. From Table VI the following observations were considered important: (a) no significant differences were observed in the activity of NR between fertilizer levels, however, activity levels tended to be higher with increased fertility, (b) all varieties exhibited maximum activity levels on April 21, when compared to other sampling dates, (c) both Nebraska Selections exhibited higher NR levels in response to higher fertility levels when compared to other varieties, (d) in both fertility levels the patterns of NR activity for all varieties were similar. No NR activity was observed on the last sampling date before flowering (May 21).

The seasonal patterns for protease (pH 4.0) activity before the flowering stage were observed to be significantly different among varieties, dates and variety date interaction, but not between fertilizer treatments. All varieties in both fertility levels exhibited increased protease activity level patterns after April 21, the date on which the decline of NR activity began. Higher protease levels were observed in Triumph 64 before the flowering stage when compared to high grain protein wheat varieties. The increased protease activity levels in Triumph 64 could be the result of its early maturity.

The pH 7.0 protease levels were not different, either due to fertilizer levels, or varieties. The pattern of both proteases (pH 4.0 and 7.0) were similar in both the fertility levels. Both proteases exhibited an increasing trend in activity levels after April 21.

Water soluble protein content, protein percent and nitrate contents of the four wheat varieties are graphically shown in Figure 9. Water soluble protein contents before the flowering stage were not

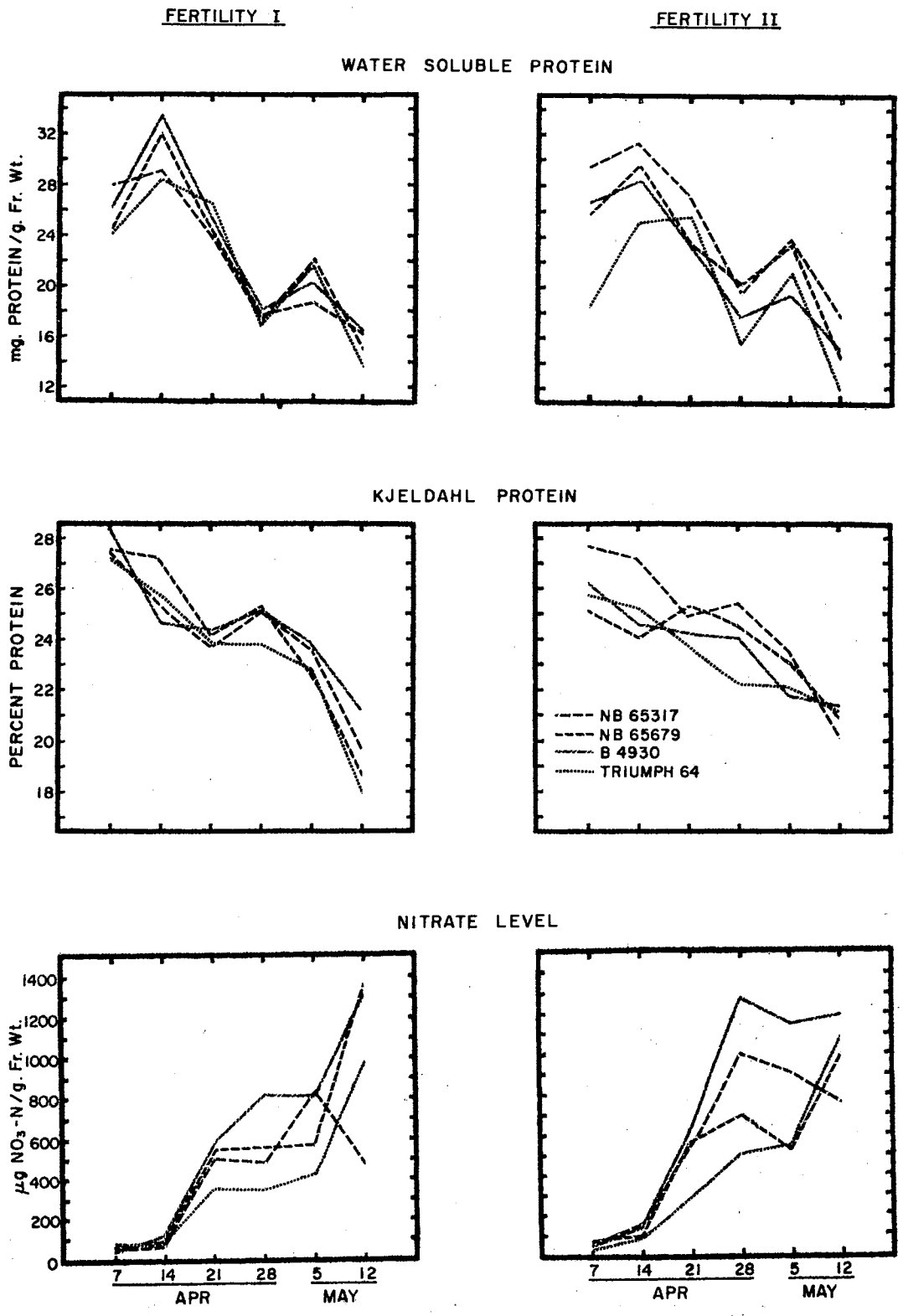

Figure 9. Patterns of leaf water soluble protein, percent Kjeldahl protein and nitrate content before the flowering stage for wheat. Fertility I (60 lb.) and Fertility II (120 lb.) N/A. was applied April 5, 1970.

different among varieties nor fertility levels; however, differences were observed among sampling dates. NB65317 exhibited a slightly higher water soluble protein content than the other varieties in response to the additional fertilizer.

Nitrate nitrogen content of the four wheat varieties, as shown in Figure 9 was not significantly different at the two fertility levels, whereas, varieties and dates exhibited highly significant differences. However, there was a general trend for nitrate content to be higher with higher nitrogen application. All varieties except NB65317 exhibited an increasing trend of leaf nitrate content in both fertility levels until the last date of sampling before the flowering stage. The high grain-protein wheats on the average had higher levels of nitrates throughout the season at both fertility levels than did Triumph 64.

Leaf Kjeldahl protein percents of four wheat varieties for both the fertility levels are presented in Figure 9, and the analysis of variance in Table VI. There were no significant differences among varieties nor fertility levels. The only differences in leaf protein percent were observed for sampling dates. In general, protein percent declined as the plant approached physiological maturity. Additional fertilizer, however, tended to slow down the decline in protein percent. This may be a reflection of slightly increased NR activity at the higher fertility level.

Activity levels after the flowering stage

Starting on May 21, plant samples were analyzed separately as middle and top leaves of the plant, for proteases, water soluble

protein, and protein percent. On May 28 only top leaves of the plant were analyzed since the lower and middle leaves of the plant were yellowing.

Average means for both dates for proteases, water soluble protein and protein percent are presented in Table VII. On May 21, protease (pH 4.0) levels were different among varieties, leaf position, and variety X position of leaf interaction, whereas, protease (pH 7.0) was different only in leaf position. All varieties exhibited higher activity levels in the top leaves compared to the middle leaves. Protease activity tended to be higher in the high soil fertility than lower fertility level. Both Nebraska Selections had higher levels of activity than B4930; whereas, Triumph 64 was very low. A similar pattern in the activity levels was observed even on the last date of sampling (May 28). In general, protease activity levels were low in the middle leaves of the plant, with a significant decrease observed between the last two sampling dates on the top leaves.

Water soluble protein contents were different among varieties, position of leaf and dates after the flowering stage. Higher fertility levels did not influence the water soluble protein content in the middle leaves; however, the top leaves responded to higher fertility levels with increased water soluble protein. All the high grain-protein wheats exhibited higher levels of water soluble protein than did Triumph 64. A rapid decline in the water soluble protein content was observed in the top leaves of the plant on the last two sampling dates.

No significant differences in protein percent (Kjeldahl) were observed within the middle and top leaves among varieties nor between

TABLE VII

SEASONAL AVERAGES AFTER FLOWERING FOR LEAF PROTEASES, WATER SOLUBLE PROTEIN AND PERCENT PROTEIN OF 4 WHEAT VARIETIES (1969-70)

Varieties and dates	Position of leaves	Protease (4.0) mgs. protein digested/g. Fr. Wt. 60# N	120# N	Protease (7.0) mgs. protein digested/g. Fr. Wt. 60# N	120# N	W.S. protein mgs./g. Fr. Wt. 60# N	120# N	Percent protein 60# N	120# N
NB65317									
May 21	Middle	3.31	3.49	0.36	0.78	2.48	2.72	7.28	8.87
	Top	12.65	13.50	1.60	2.05	12.33	19 58	12.96	16.81
May 28	Top	7.54	11.95	1.29	1.54	6.43	7.99	9.46	11.71
NB65679									
May 21	Middle	1.72	2.58	0.24	0.17	1.57	2.96	7.34	8.62
	Top	12.76	13.64	1.72	1.99	11.02	16.12	12.62	16.43
May 28	Top	8.17	11.82	1.16	0.65	5.22	8.15	9.34	8.72
B4930									
May 21	Middle	2.15	1.82	0.36	0.80	2.96	5.28	9.46	9.74
	Top	13.20	13.40	2.25	1.82	12.23	16.82	15.71	16.62
May 28	Top	6.69	8.14	1.37	0.70	4.85	6.53	11.93	11.74
Triumph 64									
May 21	Middle	1.56	1.46	0.22	0.89	2.54	2.75	11.30	13.74
	Top	6.17	8.67	1.65	1.44	3.56	5.90	12.37	14.77
May 28	Top	5.35	6.09	1.15	0.62	5.51	5.18	11.65	11.52

fertilizer levels. A rapid decline in the protein percent in the top leaves of the high grain-protein wheats on the last two sampling dates was observed; whereas, Triumph 64 did not exhibit this trend: This rapid decline in the protein percent could be a reflection of protease activity at this time, which was still high in the high grain-protein wheats. On May 21, the Nebraska Selections had higher protein percent (about 4%) in the top leaves in response to the 120# N, while the other two varieties had only about 2 percent greater protein content indicating a superior response of the Nebraska Selections to high nitrogen levels. The decline in protein percent was faster in the top leaves of high grain protein wheats than Triumph 64 between last two sampling dates. These declines in protein percent near maturity are conceivably the reflection of protease activity in the high protein wheats.

The relationship of NR, proteases, and other nitrogenous components to the grain yield, protein percent and protein production

Grain yield, percent protein and yield of grain protein per acre in both fertility levels are shown in Table VIII. With the exception of Triumph 64, increased fertility significantly increased the grain yield in all varieties. Triumph 64 showed an increase in protein percent with increased fertility level without an increase in grain yield; whereas, protein percent decreased with an increase in yield especially in NB65679 and B4930. All varieties had higher protein production per acre at the higher fertility level.

The observation presented above suggests there is a relationship between levels of NR, proteases and their activity period in the

TABLE VIII

AVERAGE GRAIN YIELD, PERCENT PROTEIN AND PROTEIN PRODUCTION PER ACRE OF 4 WHEAT VARIETIES (1969-70)

Varieties	Yield Bu/A		Percent protein		Protein in lbs/A	
	60# N	120# N	60# N	120# N	60# N	120# N
NB65317	27.55	47.20	19.5	19.0	309	540
NB65679	39.10	48.90	19.3	17.6	447	509
B4930	30.40	34.60	18.1	17.2	330	356
Triumph 64	39.60	40.95	12.8	15.6	305	380

production of grain protein per unit area. The following items appear to be of significance. Nebraska Selections exhibited increased NR and protease activity levels in response to higher fertility levels. Similar increased NR activity levels in the Nebraska Selections were also observed with increased fertility levels in the spring by Duffield (1971). A substantial increase in pounds of protein per acre (231 lbs for NB65317 and 62 lbs for NB65679) conceivably occurred in response to enhanced NR activity levels and proteases (especially after flowering stage). In general, the Nebraska Selections exhibited higher NR, proteases (after flowering stage), and increased yield and grain-protein content per acre. Johnson et al. (1968) pointed out that the high protein genetic trait is associated with more efficient translocation of nitrogen from the plant to the grain. Protease activity levels in the Nebraska Selections were higher on some dates in this study, and could conceivably enhance greater breakdown of protein in the green parts of the plant for translocation to the seed.

The protein contents are high in all samples and appear to be the result of the large soil nitrogen supply plus the high added nitrogen. Johnson et al. in 1968 indicated that there are threshold levels for nitrogen which influence the grain protein in high protein wheats. It is conceivable that sufficient nitrogen was applied to raise the enzyme levels in Triumph 64 to the point that there was no difference among the varieties on some sample dates. The difference in NR and proteases noted on some sample dates plus the increase in the water soluble protein contents indicate there are differences between Triumph 64 and the high grain-protein wheats. In Study 2, the limited number of replications among fertility levels, and the

relatively dry season served to contribute to increased error terms which reduced the precision of the experiment.

Information is very limited on the effects of environment on the protease enzyme as well as the part which the two enzymes play in the plant. The characterization data would indicate that activity levels are increased under higher temperatures and breakdown of the leaf proteins would be stepped up by hot weather. The general pH of the cell would not appear to be down to the pH 4.0 level on the basis of the buffering capacity needed to maintain a pH of 7.0 in the homogenizing media; however, it is not inconceivable that localized areas within certain membranes of the cell could have lower pH levels than are present on the average. Also it is recognized that activity levels obtained in vitro may not represent the in vivo situation. Substrate, temperature and buffering are at near optimum conditions in the assay and this probably is not true in the plant cell.

The function of the two protease enzymes are not clear at the present time. The marked difference in response to pH definitely would support two enzyme complexes. The amino acid data would support the pH 7.0 complex being an exopeptidase, i.e., cleaving single amino acids from the ends of the peptide. The pH 4.0 protease could conceivably be an endopeptidase on the basis of the large protein degradation and low amino acid release, i.e., cleaving of peptide units at random in the protein.

A number of points remain to be clarified. The relationship and importance of the two enzyme forms remains to be established and clarified. Better substrate conditions need to be established in order to improve assay conditions. Hemoglobin, an animal protein, would not

appear to be the best substrate. A better substrate might be a form of purified or prepared protein such as polyamino acid where the molecular weight is known. Purified gluten or other seed protein should be examined as a potential substrate.

The question of the genetic control of the protease needs to be clarified. Hopefully a system could be developed to permit a genetic analysis for use in breeding work. The present methodology is much too slow to permit working with a large number of samples needed for such a study. Potential means of improving efficiency would be to measure the digested protein spectrophotometrically, which would be much faster than the Lowry procedure.

While the NR and protease levels influence the water soluble protein levels in the plant, the translocation of plant constituents removes part of the protein and thus not only protease, but the other factors affecting protein levels in the leaves and thus preclude a close relationship among these factors at all times.

CHAPTER V

SUMMARY AND CONCLUSION

The objective of these studies were to determine: (a) characterization of protease present in the green leaves of the wheat plant; (b) the activity levels of protease during early stages of germination and its influence on the production of free amino acids, tryptophan, IAA and growth in 'high' and 'low' protein wheats; (c) the seasonal patterns of nitrate reductase and protease and their relationship in the nitrogenous compounds in the green leaves as well as grain yield and protein content of grain; and (d) the effect of added nitrogen fertilizer on the levels of nitrate reductase, protease and their influence on the nitrogenous components in the green leaves as well as on the yield and grain protein production.

The following results were found:

Protease in the green leaves of the wheat plant is a complex of two enzymes which are of exo and endo in nature on the basis of activity levels at pH 7.0 and 4.0 respectively, and the amino acid release pattern.

A growth chamber study was conducted to observe the protease activity levels between NB65317 and Triumph 64 (high and low protein wheats respectively) during early stages of growth. NB65317, a high grain-protein wheat, exhibited higher levels of protease activity resulting in increased amounts of free amino acids, tryptophan, IAA

and growth when compared to Triumph 64, a low protein wheat.

In the field studies 'high' grain protein wheats exhibited higher levels of nitrate reductase throughout the growing season and higher protease levels especially after the flag leaf stage. The trend of protease activity in Triumph 64 was different from that of other varieties and could be the reflection of its early maturity.

An inverse relationship was found to exist between nitrate reductase and protease activity. Water soluble protein content was positively correlated with the activity levels of NR in the early spring growth, and with protease near flowering and physiological maturity stages of the plant.

Higher nitrate reductase and protease levels were associated with the increase in grain yield and grain protein production per acre.

Added nitrogen fertility in Study 2, markedly increased the levels of nitrate reductase, water soluble protein and protein percent in the Nebraska Selections. No differences in protease with added nitrogen were observed until the flowering stage, but later the protease levels increased and the activity period was extended in the Nebraska Selections. Grain yield and protein production per acre were increased in Nebraska Selections. A substantial increase in pounds of protein per acre (231 lbs for NB65317 and 62 lbs for NB56579) conceivably occurred in response to enhanced nitrate reductase levels and higher and prolonged protease levels in response to added nitrogen.

I believe that it is possible using genetic material which has the ability to take up more nutrients or else to translocate a greater proportion of these nutrients from the vegetative plant parts to the seed to develop varieties which in turn will have higher protein

content, be more nutritious, and serve to make more efficient use of the nitrogen which is added to the soil.

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